Prevalence of tetrahydrobiopterine (BH4)-responsive alleles among Austrian patients with PAH deficiency: comprehensive results from molecular analysis in 147 patients

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ORIGINAL ARTICLE

Prevalence of tetrahydrobiopterine (BH4)-responsive alleles among Austrian patients with PAH deficiency: comprehensive results from molecular analysis in 147 patients

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Abstract Phenylketonuria (PKU, MIM 261600) is an autosomal recessive disorder caused by mutations of the phenylalanine hydroxylase gene (PAH, GenBank U49897.1, RefSeq NM_000277). To date more than 560 variants of the PAH gene have been identified. In Europe there is regional distribution of specific mutations. Due to recent progress in chaperone therapy, the prevalence of BH4-responsive alleles gained therapeutic importance. Here we report the mutational spectrum of PAH deficiency in 147 unrelated Austrian families. Overall mutation detection rate was 98.6 %. There was a total of 62 disease-causing mutations, including five novel mutations IVS4+6T>A, p.H290Y, IVS8-2A>G, p.A322V and p.I421S. The five most prevalent mutations found in patients were p.R408W, IVS12+1G>A, p.R261Q, p.R158Q and IVS2+5G>C. Neonatal phenylalanine levels before treatment were available in 114/147 patients. Prediction of BH4-responsiveness in patients with full genotypes was exclusively made according to published data.

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Among the 133 patients needing dietary treatment, 28.4 % are expected to be BH4 "non-responsive", 4.5 % are highly likely BH4-responsive, 35.8 % are probably BH4-responsive while no interpretation was possible for 31.3 %. The mutation data reflect the population history of Austria and provide information on the likely proportion of Austrian PKU patients that may benefit from BH4-therapy.

Introduction

Phenylketonuria (PKU, MIM 261600) is an autosomal recessive disorder caused by alterations of the *PAH* gene (NM_000277) coding for L-phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1). The *PAH* gene covers 120 kb on chromosome 12q23.2 (DiLella et al. 1986). More than 560 different gene variants are listed in the PAH mutation database at www.pahdb.mcgill.ca (Scriver et al. 2003). In most Central and Northern European countries 3–5 mutations account for more than 50 % of PKU mutations in the population. The majority (60 %) of mutations are missense mutations that cause reduced or absent enzyme function by a variety of pathogenetic mechanisms. Recently it has been shown that effects on protein folding and degradation play a major pathogenetic role (Pey et al. 2007; Scriver 2007; Gersting et al. 2008). These effects can be studied by in vitro expression studies (Erlandsen and Stevens 2001; Pey et al. 2003) and in the in vivo mouse models $Pah^{enu1/1}$ (Gersting et al. 2010) and $Pah^{enu1/2}$ (Lagler et al. 2010). Phenylalanine hydroxylase function and response to BH4-administration result from an interplay between genotype, metabolic state and cofactor concentration (Staudigl et al. 2011). Null mutations that completely abolish protein function include nonsense mutations (5 % of recorded mutations), splice-site mutations (11 %), small deletions (13 %), and small insertions (1 %). Large deletions are generally rare (Møller et al. 2007) but have been reported with a frequency of 3 % within Slavic population (Kozak et al. 2006).

Most individuals affected with PKU are compound heterozygotes carrying different mutations on both alleles. In general, the mutation with higher residual activity determines the overall enzyme function in an individual patient (Gulderberg et al. 1998; Zurflüh et al. 2008); a hypomorphic mutation is described as "functionally hemizygous" if there is a null mutation on the other allele. Knowledge of the functional effects of two mutations allows a prediction of the likely residual enzyme function even in patients with novel genotype combination, but there are still unknown factors that influence the phenotype particularly in patients with mild or moderate forms of PKU (Kayaalp et al. 1997; Gulderberg et al. 1998).

The effect of BH4 responsiveness for some *PAH* missense mutations has first been reported in 1999 (Kure et al. 1999). A range of mechanisms is thought to contribute to this effect, including chaperon-like activity of BH4 by preventing protein misfolding and protection from inactivation of the mutant protein (Pey et al. 2004), increasing *PAH* gene expression (Munk-Martin and Hyland 2001), stabilization of misfolded protein preventing premature decay of the misfolded protein (Gersting et al. 2008) and increase of decreased BH4 binding affinity of K_m-variants of the PAH enzyme (Erlandsen and Stevens 2001). The BIOPKU database www.bio-pku.org provides preliminary information on BH4 responsiveness of mutated PAH proteins collected in a non-standardized fashion on a multicenter level. Proportion of patients with BH4 responsive PKU based on BH4 tests was found to be 85 % (91/ 107) in Italy (Fiori et al. 2005), 37 % (11/31) in Spain (Desviat et al. 2004), 46 % (17/37) in South Wales (Mitchell et al. 2005), and in the Netherlands (86/186) (Anjema et al. 2011). Retrospective analysis of data on 1919 patients who underwent a BH4 loading test between 1988–2002 indicated that 70 % of patients with neonatal phenylalanine levels <800 μmol/l were found to be BH4-responders (Bernegger and Blau 2002).

In most European countries the spectrum of PKU mutations in representative patient cohorts was determined in the 1990s (Zschocke 2003) but there are as yet no such data for Austria. Here we report comprehensive mutation analysis in a large cohort of PKU patients treated in the four metabolic centers of Austria. Results reflect the history of Austria as a central European country with strong links to Eastern Europe and allow an estimation of the likely proportion of patients that may benefit from BH4 therapy.

Material and methods

Genotype analysis of Austrian patients with PAH deficiency started in 2004 at the laboratory of metabolic diseases, Department of Pediatrics, Medical University of Graz and since then was offered to all patients treated at the four Austrian metabolic centers. The incidence of PKU in Austria is approximately 1:12,000 (Kasper et al. 2010).

Subjects

A total of 168 patients from 147 unrelated families were analysed. Patient numbers per center were as follows: Vienna 89, Graz 61, Salzburg 7, Innsbruck 11. Elevated phenylalanine concentrations were detected by newborn screening and confirmed by quantitative analysis of plasma aminoacids. Analysis of urinary pterins and dihydropterine reductase assays were performed to exclude BH4 deficiency (Nenad Blau, Zurich, Switzerland). For molecular studies, 3–5 ml of

EDTA blood was drawn as part of regular blood sampling after having obtained written informed consent of patients or caregivers.

Methods

DNA-extraction was done from EDTA blood samples using standard methods (QIAamp DNA Blood Mini Kit, QIAGEN, Vienna, Austria). Over a period of 8 years (2004–2011) three different methods were used with an over all detection rate of 98.6 %. Initially, restriction fragment length polymorphism analysis (RFLP) for eight of the most frequent mutations of *PAH* gene (GenBank U49897.1, RefSeq NM_000277) previously found in central Europe p.R408W (c.1222C>T), p.R261Q (c.782G>A), p.Y414C (c.1241A>G), p.R158Q (c.473G>A), IVS10-11C>T (c.1066-11G>A), IVS12+1G>A (c.1315+1G>A), p.P281L (c.842C>T), p.I65T (c.194T>C) and for several frequent polymorphisms p.Q232Q (c.696A>G), p.V245V (c.735G>A), p.L385L (c.1155G>C), IVS3-22C>T (c.353-22C>T) was performed; this method led to the characterization of mutations on both alleles in 30 patients. In the remaining samples mutation scanning was performed by melting point (T_m) analysis using SYBR Green-labeled PCR replicons of 100 bp-fragments, overlapping at a length of 12 bp, visualized in a ROCHE Light Cycler. Replicons with aberrant melting points were sequenced in 21 cases. Subsequently (12/2004 to 12/2005) denaturing gradient gel electrophoresis (DGGE) was used for mutation scanning in another 29 patients following a previously published protocol (Zschocke et al. 2000). Since January 2006 another 67 samples were analyzed by complete sequencing of all exons and 100 bp of flanking introns of the *PAH* gene.

Each of the DNA alterations in all 147 patients claimed as a pathogenic change was confirmed by RFLP analysis.

The rarity of novel mutations was confirmed by comparison with at least 110 wild type alleles. *In silico*-prediction of pathogenicity of novel missense mutations was done with the PolyPhen-2 bioinformational tool (http://genetics.bwh.harvard.edu/pph2/) for the HumVar score and alignment analysis. Splice site mutations were analyzed with SplicePort (spliceport.cs.umd.edu) using a threshold of −0.5 for the change of FGA (feature generation algorithm) score (www.cbs.dtu.dk).

Four patients in whom we had only identified one mutation underwent additional MLPA (multiplex ligation-dependet probe amplification) analysis (Zschocke, data not shown).

According to detailed information provided in the literature (Pey et al. 2003 and 2004; Blau and Erlandsen 2004; Zurflüh et al. 2008; Karacic et al. 2009) the genotype combination was assigned to one of four groups:

1) "non BH4 responsive" if both alleles showed null mutations
2) "BH4 responsive" if the patient carried a consistently BH4 responsive mutation on at least one allele
3) "probably BH4 responsive" in the presence of at least one mutation encoding a protein with known residual activity but inconsistent information on BH4 response

Table 1 The 13 most prevalent mutations (GenBank U49897.1, NM_000277) detected in 147 Austrian Phenylketonuria (PKU; MIM 261600) patients

Mutation	Trivial name	No. (percentage) of alleles
c.1222C>T	p.R408W	67 (22.8 %)
c.1315+1G>A	IVS12+1G>A	34 (11.6 %)
c.782G>A	p.R261Q	20 (6.8 %)
c.473G>A	p.R158Q	13 (4.4 %)
c.168+5G>C	IVS2+5G>C	12 (4.1 %)
c.1241A>G	p.Y414C	11 (3.7 %)
c.1066-11G>A	IVS10-11G>A	11 (3.7 %)
c.1169A>G	p.E390G	10 (3.4 %)
c.842C>T	p.P281L	10 (3.4 %)
c.1066-3C>T	IVS10-3C>T	8 (2.7 %)
c.165delT	p.F55fs	7 (2.4 %)
c.143T>C	p.L48S	5 (1.7 %)
c.1208C>T	p.A403V	5 (1.7 %)
Total:		213 (72.4 %)

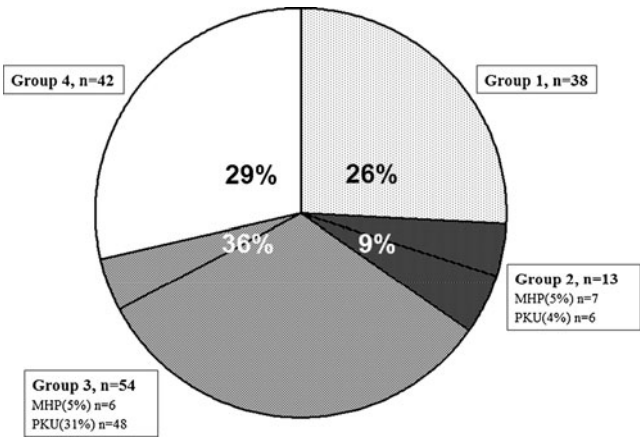

Fig. 1 Prediction of BH4 responsiveness according to full genotype in our cohort of 147 Austrian patients with PAH deficiency (134 PKU, 13 MHP) according to published data (Pey et al. 2003 and 2004; Blau and Erlandsen 2004; Zurflüh et al. 2008; Karacic et al. 2009). Group 1: non BH4 responsive as both alleles show null mutations. Group 2: BH4 responsive as at least one allele is known to be consistent with BH4 responsiveness. Group 3: probably BH4 responsive as at least one mutation is coding for a protein with known residual enzyme activity but inconsistent information about BH4 responsiveness. Group 4: unknown as information on residual activity or BH4 responsiveness is pending. MHP- mild hyperphenylalaninaemia without the need of dietary restriction; PKU- dietary restriction of daily phenylalanine intake

4) "unknown" if information on residual activity or BH4 responsiveness of at least one allele was pending

Patients were either classified as PKU or mild hyperphenylalaninaemia (MHP) by the need of dietary restriction of daily phenylalanine intake versus a free diet according to information provided by the referring centers.

Results

Of the 147 individuals included in this analysis, 134 were classified as PKU, while 13 were MHP. Full genotype, phenylalanine levels before diet, actual patient age as well as population background are shown in supplementary Table 1.

Disease causing mutations were identified on 290 alleles (98.6 %). We were able to detect both mutations in 143 patients while in four patients only one mutation could be identified, despite additional MLPA analysis. Among our cohort a total of 62 different mutations were identified; 13 mutations with an allele frequency ≥1.7 % are shown in Table 1. Fourty nine mutations had an allele frequency of less than 1.7 %; of these 31 mutations were detected on single alleles (suppl. Table 1).

Thirty eight patients carried known null mutations on both alleles, excluding BH4 responsiveness in 26 % of this cohort (group 1) (Fig. 1). Thirteen patients carried one of the following four mutations that have been reported with consistent BH4 responsiveness p.D129G (c.386A>G), p.A300S (c.898G>T), p.I306V (c.916A>G) and p.E390G (c.1169A>G), on at least one allele, indicating a high probability of BH4 responsiveness in 9 % of our cohort (group 2).

Fifty four patients carried mutations with residual activity on at least one allele accounting for 36 % of our cohort (group 3), 42 patients were carrying mutations of unknown residual activity on both alleles or in functional hemizygosity representing 29 % of this cohort (group 4). Of the four patients with an unidentified mutation on the second allele one could be assigned to group 2 while three had to be assigned to group 4.

Novel mutations

Five novel mutations were detected in seven patients in our cohort of patients: IVS4+6T>A (c.441+6T>A), p.H290Y (c.868C>T), IVS8-2A>G (c.913-2A>G), p.A322V (c.965C>T) and p.I421S (c.1262T>G). They all were found in patients of central European origin. Family segregation was consistent with autosomal recessive inheritance and the respective sequence alteration could not be detected in 112 control samples of central European origin. Details on in silico and splice port analysis, full genotype and phenotype are provided in Table 2.

Discussion

Our study cohort consisted of 147 unrelated Austrian families (168 patients), representing about 40 % of all patients with PAH deficiency diagnosed in Austria since 1967. Of this cohort 24 (16 %) had a recent migration background. Within the 294 alleles analysed we were able to identify 290 disease causing mutations. This reflects a detection rate of 98.6 %.

In Austria five mutations account for 49.6 % of investigated alleles. This is in line with other Central European

Table 2 Novel mutations of the *PAH*-Gene (GenBank U49897.1, NM_000277)

cDNA	Gene product	In silico analysis (PolyPhen2)			Other allele	Phenotype
		HumVar Score	Conserved (%)	Classification		
a) Missense mutations						
c.868C>T	p.His290Tyr (p.H290Y)	1.0	119 of 120 (99 %)	Possibly damaging	p.R408W	PKU
c.965C>T	p.Ala322Val (p.A322V)	0.87	72 of 106 (68 %)	Possibly damaging	p.R408W	Mild PKU (vegetarian diet)
c.1262T>G	p.Ile421Ser (p.I421S)	0.948	Ile: 33 of 91 (36 %) Val: 58 of 91 (64 %)	Possibly damaging	p.F39L	PKU

cDNA	Intron	Splice Port (FGA threshold −0.5) FGA score wild type	FGA score mutant allele	Other allele	Phenotype
b) Splice site mutations					
c.913-2A>G	8	0.89	Below threshold	p.R408W	PKU
c.913-2A>G	8	0.89	Below threshold	p.R158Q	PKU
c.913-2A>G	8	0.89	Below threshold	IVS12+1G>A	PKU
c.441+6T>A	4	0.83	−0.28	p.P281.L	PKU

countries. In Northern European countries few mutations account for high allele frequencies, e.g p.R408W (c.1222C>T) in Baltic States (76 % in Latvia, 84 % in Estonia) and p.R408W (c.1222C>T), p.Y414C (c.1241A>G), IVS12+1G>A (c.1315+1G>A) in Scandinavia. Southern Europe is more heterogeneous and the most frequent mutations, e.g., the IVS10-11G>A (c.1066-11G>A) only covers for up to 19 % in Southern Italy, 15 % in Spain, 13 % in Greece (Zschocke 2003).

The most frequent mutation identified among our cohort was p.R408W (c.1222C>T) with an overall allele frequency of 23 %. It is the most prevalent mutation worldwide but its allele frequency varies considerably within Europe in a Northeast-Southwest devide: up to 84 % in the Baltic States (Lilleväli et al. 1996; Pronina et al. 2003) to about 50 % in Poland, Czech Republic and Slovakia (Jaruzelska et al. 1993; Kadasi et al. 1995; Kozak et al. 1997), 36 % in Croatia (Karacic et al. 2009) and less than 5 % in Southern Europe (Italy, Greece, Spain), and complete absence in Moroccan PKU patients (Dahri et al. 2010).

IVS12+1G>A (c.1315+1G>A) was found on 12 % of all alleles investigated in this study. This mutation is prevalent in Denmark (37 %) (Guldberg et al. 1993), Belgium and Netherlands (24 %) and Scandinavia, e.g., Norway (15 %) (Eiken et al. 1996).

p.R261Q (c.782G>A) accounted for 7 % of alleles, similar to percentages found in Germany (6 %) (Zschocke and Hoffman 1999), while it is found with markedly higher prevalence of 32 % in Switzerland and 16 % in South Italy (Daniele et al. 2007).

p.Y414C (c.1241A>G) was found in only 3.7 % of our 294 alleles, which is below the frequency of approximately 10-18 % in Northern Europe and above the frequency of 1-3 % in Southern Europe (Zschocke 2003).

Austrian results represent migrational shifts as well as genetic heterogeneity among a rather small country. While Slavonian heritage is strongly reflected by the high prevalence of p.R408W (c.1222C>T) there is lower prevalence of mutations which are common in Italy or among the South Western Europe mutational spectrum. Prevalence of more frequent Turkish and South Eastern Europe mutations reflect migrational shifts that took place over the last decades (Fig. 2).

A total of five novel mutations were identified in seven patients: IVS8-2A>G (c.913-2A>G) $n=3$, IVS4+6T>A (c.441+6T>A), p.H290Y (c.868C>T), p.A322V (c.965C>T), p.I421S (c.1262T>G).

Among the cohort of 147 patients 134 patients had PKU while 13 patients (9.4 %) had MHP and were thus not expected to benefit from BH4-treatment. In this cohort the following eight mutations are assumed to determine the MHP phenotype in compound heterozygosity: p.A104D (c.473G>A), p.D129G (c.386A>G), p.V177L (c.529G>C), p.V245A (c.734T>C), p.A300S (c.898G>T), p.I306V (c.916A>G), p.E390G (c.1169a>G), p.A403V (c.1208C>T). According to published

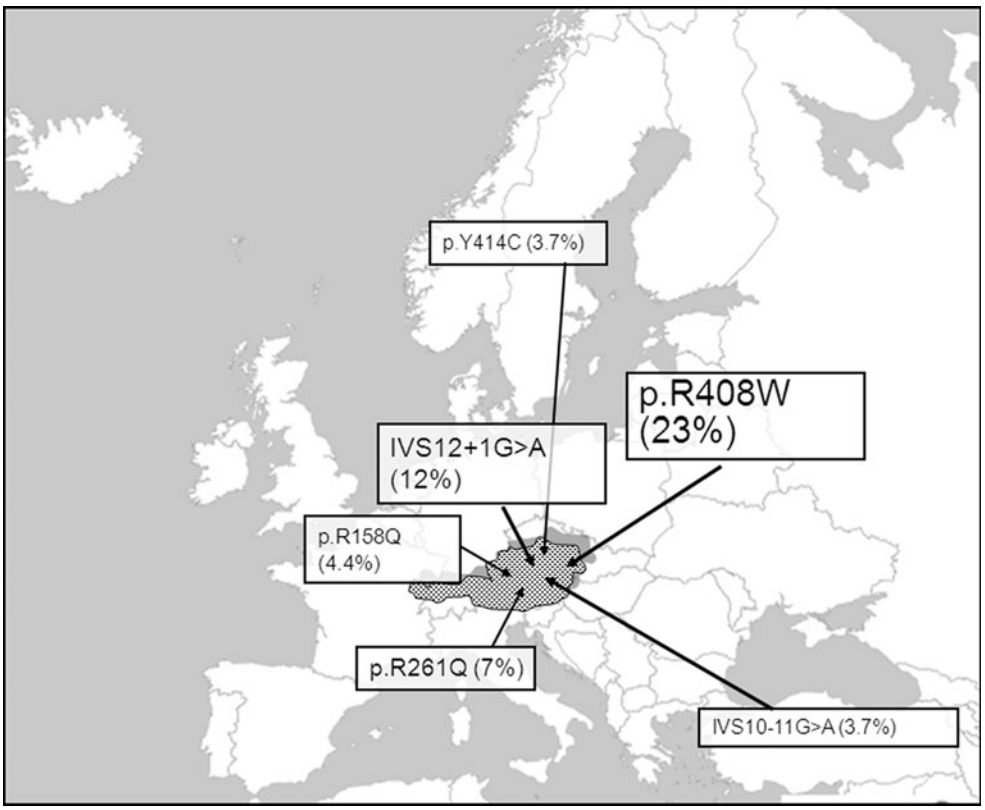

Fig. 2 Allele frequency of common mutations found in Austrian patients with PAH deficiency within the European context. Possible origins are indicated by the position of the textbox on the map and allele frequency in Austria is given in brackets

data on BH4 responsiveness seven of our MHP patients would have been assigned to group 2 ("BH4 responsive") and six to group 3 ("probably BH4 responsive").

Among the cohort of 134 PKU patients prediction of BH4 responsiveness was possible in 33 %. This prediction is most reliable for the 28.4 % of PKU patients with null mutations on both alleles. All PKU patients with a genotype predicting a high likelihood of BH4 responsiveness (4.5 %, n=6) were carrying the mutation p.E390G in compound heterozygosity. The minor chance of carrying an undetected unresponsive mutation in cis was excluded through the analysis of all coding exons by sequencing or DGGE/T_m analysis. 35.8% carried mutations with residual activity on at least one allele with some chance of BH4-resonsiveness. No prediction of the likelihood of BH4 responsiveness could be made for 31.3 % of PKU. Remarkably the largest group (35.8 %) comprises patients with mutations that have been classified inconsistently in different studies. There are many reasons to explain these discrepancies that can be categorized as follows: i) modifiers of residual PAH activity like interallelic complementation in compound heterozygous genotypes (Leandro et al. 2006) or discrepancies in the folding or quality control machineries (Dipple and McCabe 2000; Pey et al. 2004); ii) inconsistencies of test protocols and definitions (Zurflüh et al. 2008); or iii) false test results, e.g., due to the impact of intra- and interindividual variability of intestinal BH4 absorption and blood phe levels (Zurflüh et al. 2008; Trefz et al. 2009). This illustrates that the identification of BH4 responsive patients cannot be based on mutational analysis alone. Better harmonization of BH4 loading tests is needed and the consideration of alternative test methods like challenge with labeled or unlabeled phenylalanine may be reasonable. Currently a prospective national trial comparing the BH4 loading test as recommended by the European working group for phenylketonuria (Blau et al. 2009) followed with a standardized phenylalanine challenge is conducted with patients out of this study cohort among Austria.

By using the Hardy-Weinberg formula Zurflüh et al. in 2008, postulated a frequency of 55 % of BH4 responsiveness in European populations (range 17-79 %, lowest in Baltic Countries, highest in Spain). This number could be an overestimate for Austria as in this scenario all patients out of group 3 and one third of patients out of group 4 would have to show BH4 responsiveness. A standardized BH4 loading test remains essential to finally assess BH4 responsiveness.

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Conflict of interest None.

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